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Differential alveolar epithelial injury and protein expression in pneumococcal pneumonia

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ABSTRACT

The integrity of the alveolar epithelium is a key factor in the outcome of acute lung injury. Here, we investigate alveolar epithelial injury and the expression of epithelial-selective markers in *Streptococcus pneumoniae*-induced acute lung injury. *S. pneumoniae* was instilled into rat lungs and alveolar type I (RTI_{40}/podoplanin, MMC6 antigen) and alveolar type II (MMC4 antigen, surfactant protein D, pro-surfactant protein C, $RTII_{70}$) cell markers were quantified in lavage fluid and lung tissue at 24 and 72 hours. The alveolar epithelium was also examined using electron, confocal, and light microscopy. *S. pneumoniae* induced an acute inflammatory response as assessed by increased total protein, SP-D, and neutrophils in lavage fluid. Biochemical and morphological studies demonstrated morphologic injury to type II cells but not type I cells. In particular, the expression of RTI_{40}/podoplanin was dramatically reduced, on the surface of type I cells, in the absence of morphologic injury. These data demonstrate that type II cell damage can occur in the absence of type I cell injury without affecting the ability of the lung to return to a normal morphology. These data also demonstrate that RTI_{40}/podoplanin is not a type I cell phenotypic marker in experimental acute lung injury caused by *S. pneumoniae*. Given that RTI_{40}/podoplanin is an endogenous ligand for the C-type lectin receptor and this receptor plays a role in platelet aggregation and neutrophil activation, we hypothesize that the reduction of RTI_{40}/podoplanin on type I cells might be important for the regulation of platelet and/or neutrophil function in experimental acute lung injury.

KEYWORDS lung, pneumonia, podoplanin

INTRODUCTION

Streptococcus pneumoniae remains the commonest pathogen responsible for CAP [1, 2], causing more deaths from invasive infection than any other bacterium, and being the fifth leading cause of death worldwide [3]. *S. pneumoniae* typically causes acute inflammation of lung parenchyma, characterized by alveolar exudation and influx of neutrophils, with subsequent resolution of inflammation driven by interactions between alveolar macrophages and apoptotic neutrophils [4, 5]. While much is known about these processes, surprisingly little is known about the response of alveolar epithelial cells (AECs)—critical for gas exchange and surfactant production—in acute pneumonia. Much of the information about the response of the alveolar epithelium to *S. pneumoniae* is derived from histological and ultrastructural studies [6–8], and partly reflects a lack of detectable markers specific to type I (ATI) and type II (ATII) AECs.

This is compounded by the fact that in vitro studies of the effect/s of infection on AECs are lacking

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because it is difficult to extract and maintain healthy primary ATI cells for any length of time in culture, and representative cell lines are lacking.

Maintenance and/or restoration of AEC integrity are of central importance to recovery in lung injury [9]. Highly specialized, flattened ATI cells, along with pulmonary endothelial cells, form the alveolar-capillary membrane, while ATII cells secrete surfactant and can differentiate into new ATI cells following alveolar damage [10, 11]. Given the fundamental importance of AECs to the integrity of the air-blood barrier, and the importance of rapid repair following injury, a greater understanding of the response of AECs in pneumococcal pneumonia will provide new data about the pathogenesis of disease, while helping to identify potential diagnostic tools and therapeutic targets. McElroy et al. have previously identified and studied markers of ATI and ATII cells in acute inflammatory lung injury [11–14]. The present study aimed to characterize the extent of AEC damage in a rat model of *S. pneumoniae*-induced lung injury using a range of AEC cell specific and selective markers (Table 1). Here, we demonstrate that *S. pneumoniae*-induced acute lung injury causes ATII injury but not ATI injury and that the ATI marker protein (RTI_{40}/podoplanin) is down-regulated on the surface of ATI cells without evidence of morphologic injury. Given that RTI_{40}/podoplanin is an endogenous ligand for the C-type lectin receptor and this receptor plays a role in platelet aggregation and neutrophil activation [15], we hypothesize that the reduction of RTI_{40}/podoplanin on ATI cells might be important for the regulation of platelet and/or neutrophil function in experimental acute lung injury.

METHODS

Study Animals

All experiments were in accordance with the UK Home Office Animals (Scientific Procedures) Act, 1986. Animals used were male Sprague Dawley, Specific Pathogen Free (SPF) rats. Rats were included in experiments based on normal clinical signs and body weight (*ca* 300–350 g).

Bacterial Strains and Culture Conditions

S. pneumoniae laboratory strain D39 [20] was streaked out from glycerol stocks and 1 colony was cultured for 16 hours in 3 mL brain/heart infusion broth (B.D. Biosciences, Oxford, UK) without agitation at 37°C. The culture was washed with endotoxin-free phosphate buffered saline (PBS), and then resuspended in sterile PBS. Instillate inoculum was confirmed by growing the instillate on blood agar base plates. Colony-forming units (CFU) were counted after overnight incubation at 37°C.

Rat Model of Pneumonia and Preparation of Samples

S. pneumoniae was instilled into the distal airways as described previously [11, 14]. 0.5 mL of bacteria (10^8

TABLE 1 List of Primary Antibodies Used in Immunofluorescence, ELISA, and Western Blot Analysis

Cell type	Name	Location	Size	Other identified locations	Additional information
Type I AEC	RTI_{40}	Apical surface	40 kDa	Lymphatic endothelium, kidney, bone, testis, some cancers [10]	Also known as T1α protein, E11 antigen, and podoplanin; heavily glycosylated [10]
	Antigen recognized by MMC6 antibody	Apical surface	Unknown	None (unpublished, McElroy and McKechnie, 2004)	Expressed on ATI cells (unpublished, McElroy and McKechnie, 2003)
	Aquaporin 5	Apical surface	26 kDa	Bronchiolar epithelial cells [10]	Water channel [10]
Type II AEC	Surfactant protein D (SP-D)	Cytoplasmic	43 kDa	Clara cells, gastrointestinal tract, genitourinary tract, glandular tissue [16]	Hydrophilic; has antimicrobial activity [16]
	Antigen recognized by MMC4 antibody	Apical surface	117 kDa	Clara cell [17], also identified on alveolar macrophages (unpublished, Franklin and McElroy, 2004)	Identified as aminopeptidase N (unpublished, Franklin and McElroy, 2004)
	$RTII_{70}$	Apical surface	70 kDa	Unidentified	Expressed in surfactant protein-positive cells [18]
	Pro-form of surfactant protein C (Pro-SP-C)	Cytoplasmic	21 kDa	None	Pro-form of SP-C, highly hydrophobic surfactant component [19]

or 10^9 CFUs) or PBS was instilled and rats were humanely killed at 24 hours, 72 hours, or 21 days post infection. Lungs were lavaged as previously described [11, 14]. Briefly, lungs were lavaged twice with 10 mL of sterile PBS and the total volume of returned BALF recorded. The volume of returned BALF fluid was not statistically different between treated and control groups (data not shown). For protein analysis, lungs where then stored frozen at approximately –80°C until required. Lungs for histological, immunofluorescence, and electron microscopic analysis were processed as described below.

Processing of BALF

Following collection, a sample of BALF was serially diluted in PBS and bacterial numbers assessed by culture on blood agar plates as described above. Total and differential counts of BALF cells were performed, and BALF subsequently processed as described previously [11, 14]. For analysis of membrane-associated proteins (RTI_{40}/podoplanin, MMC4), an aliquot of BALF was ultracentrifuged at 500,000 *g* (Beckman Optima Ultracentrifuge, Beckman Coulter Ltd, High Wycombe, UK) and the resulting pellet resuspended in Tris buffered saline (TBS).

Lung Processing

Following dissection, whole lungs were macroscopically assessed for damage, homogenized as described previously [11, 14], and bacterial cell numbers assessed by culture on blood agar plates as described above. For microscopical analysis, lungs were fixed by intratracheal instillation of 10 mL of 4% paraformaldehyde as described previously [11, 14]. For histological analysis, lung sections were cut and stained using haematoxylin and eosin [11, 14]. For immunofluorescence studies, small cubes of tissue were fixed overnight in 30% sucrose and 4% Optimum Cutting Temperature (OCT) compound (Bayer, Newbury, UK) in PBS [11, 14]. For electron microscopic analysis, lungs were instilled with 10 mL of 4% paraformaldehyde and removed to 4% paraformaldehyde at 4°C. Small cubes were fixed in 3% glutaraldehyde buffer containing sodium cacodylate (0.1 M), post-fixed in 1% osmium tetroxide buffer containing 0.1 M sodium cacodylate, as described previously [11, 14].

Quantification of AEC Proteins

Antibodies against rat RTI_{40}/podoplanin (Dobbs), MMC6 antigen, and MMC4 antigen (McElroy) were used to analyze proteins by semi-quantitative enzyme-linked immunosorbent assay (ELISA)-based dot blot assay (Table 1). Antibodies against pro-SP-C ("NPROSP-C"), SP-D (Polyclonal 1754), and RTI_{40}/podoplanin were used in quantification of proteins by analysis of Western blots [11, 14]. An antibody against the E11 epitope of RTI_{40}/podoplanin (Wetterwald) was used to quantify proteins by direct ELISA. AEC markers were quantified relative to control samples on the same blot.

Immunofluorescence

Frozen sections were immersed in blocking buffer (2.2% fish gelatin, 1% Triton, 20% goat serum in PBS) for 15 minutes. Primary antibody (Table 1) was applied for 30 minutes, washed, then Alexa Fluor®-conjugated isotype-specific secondary antibody (Invitrogen, Paisley, UK) added for 30 minutes. Sections were treated with 0.1% To-Pro-3, washed, and mounted in Mowiol (Merck Biosciences, Nottingham, UK). Images were acquired using a confocal laser-scanning microscope (Zeiss Axiovert LSM 510) [11]. Control and *S. pneumoniae*-treated sections were prepared and analyzed in parallel (with the same confocal settings) to allow valid comparison of staining intensities. For quantification purposes, sloughing or sloughed ATII cells were quantified in a minimum of 3 large contiguous areas of specimen (up to 585 μm^2 or 4096 × 4096 pixels with the 63× objective) and were imaged as 4 × 4 contiguous fields of view using the image tiling facility of the LSM [11].

Analysis

Data are expressed as mean ± standard deviation, and were analyzed using GraphPad InStat Version 3.0 software using ANOVA with Tukey's post-test correction unless otherwise stated. Statistical significance was assigned at a *P*-value of <0.05.

RESULTS

Characterization of Pneumonia

Histology

Instillation of D39 *S. pneumoniae* induced a widespread, patchy lung injury. Histological analysis of lung tissue at 24 hours following instillation of 1×10^8 CFU D39 showed airspaces containing cells, particularly neutrophils, and fluid. At 72 hours, post-infection airspaces still contained inflammatory cells and the alveolar walls had thickened. However, by 21 days post infection the lung had returned to

normal architecture, with thin alveolar walls and clear airspaces (Supplementary Data 1 - available online).

Viable bacteria, leucocytes, and protein in BALF

Following instillation of 1×10^9 CFU D39, *S. pneumoniae* was greatly reduced in lungs and BALF by 72 hours post infection (Figure 1A). Clearance of *S. pneumonia*e was associated with an increase in BALF leukocytes (mainly due to increased neutrophils) and increased protein concentration at both 24 and 72 hours post infection (Figure 1B–D). However, both cell and protein concentrations were reduced at 72 hours post infection compared with 24 hours post infection (Figure 1B–D). These combined data indicate an acute inflammatory process at 24 hours with evidence of resolution by 72 hours post infection.

ATI Cell Analysis

Biochemical assessment of RTI_{40}/podoplanin

To determine whether the inflammatory reaction to *S. pneumoniae* was associated with injury/necrosis of alveolar type I cells (ATI), the concentration of RTI_{40}/podoplanin was determined using an ELISA-based dot blot assay. The concentration of

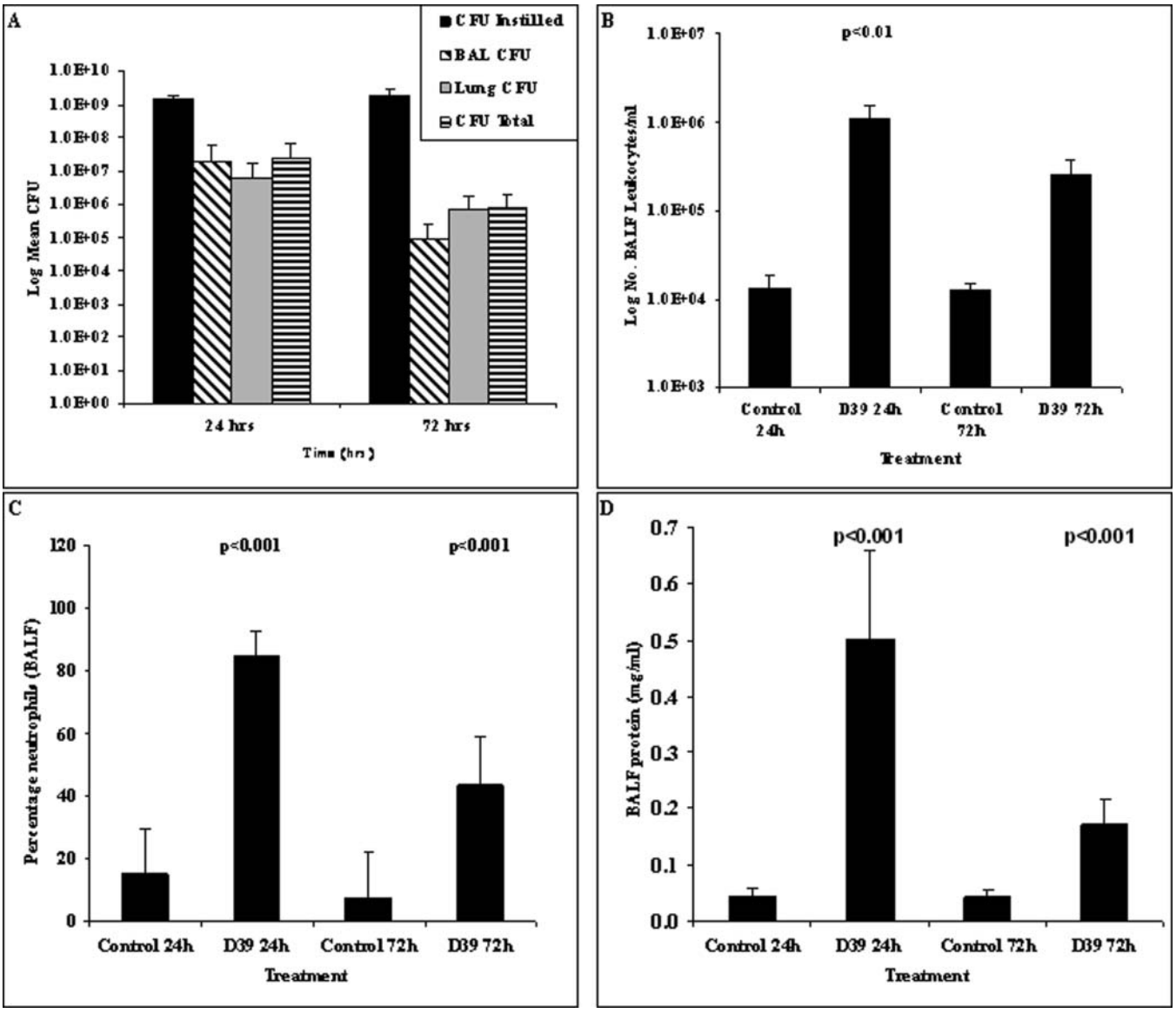

FIGURE 1 Viable *S. pneumoniae* (D39), leucocytes, and protein 24 and 72 hours post infection. Significance values shown on graphs are between control and treated only. Figure shows colony-forming units (CFU) recovered in Broncho-alveolar Lavage Fluid (BALF) and lung tissue (**A**), number of leukocytes recovered in BALF (**B**), percentage neutrophils in BALF (**C**), and BALF protein (**D**). Control 24 hours, $n = 9$; Control 72 hours, $n = 7$; D39 24 hours, $n = 5$; D39 72 hours, $n = 6$.

RTI_{40}/podoplanin in BALF and lung homogenates from animals administered *S. pneumoniae* was decreased at 24 hours post dose, compared with control values, and this decrease was dose dependent (Figure 2A). In the lung homogenates, RTI_{40}/podoplanin was decreased by approximately 85% in those animals administered 10^8 CFUs and approximately 95% in those animals administered 10^9 CFUs (Figure 2A). This decrease in lung RTI_{40}/podoplanin concentration was confirmed by Western blot analysis (Figure 2C). However, as observed with BALF protein and inflammatory cells, the decrease in lung RTI_{40}/podoplanin concentrations was less pronounced at 72 hours post dose compared with 24 hours post dose suggesting resolution/recovery (Figure 2C). Further experiments performed with a second monoclonal antibody against RTI_{40}/podoplanin in an ELISA assay (E11, [21]) confirmed the decrease in lung and BALF at 24 hours post infection (1×10^8 CFUs - data not shown).

The following morphologic studies were performed to determine whether the decrease in lung RTI_{40}/podoplanin was associated with morphologic injury to ATI cells (Figures 3 and 4)

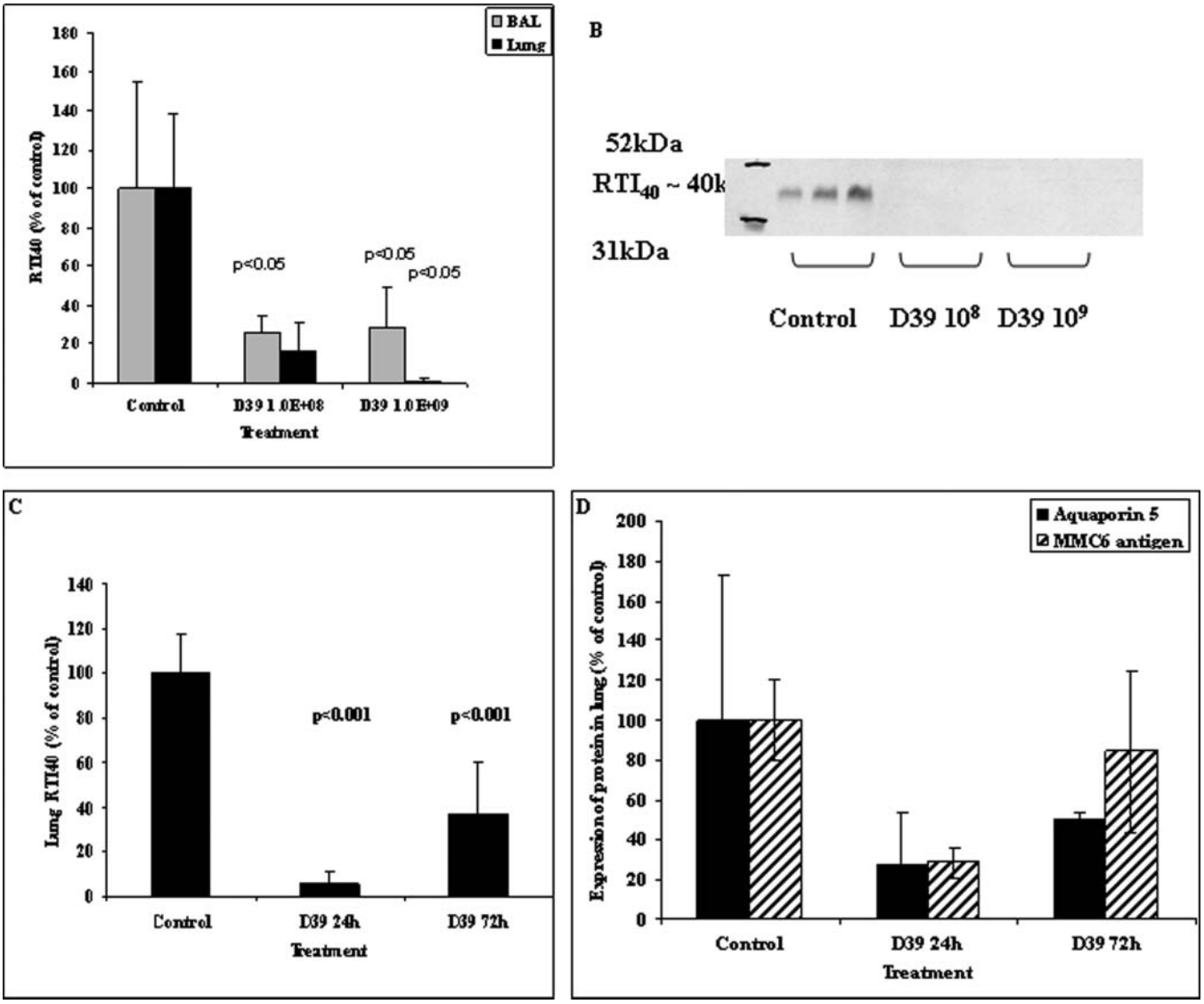

FIGURE 2 Alveolar epithelial Type I cell marker expression 24 and 72 hours post infection with *S. pneumoniae* (D39). Significance values shown on graphs are between control and treated only. Figure shows concentration of RTI_{40} in BALF and lung tissue as assessed by ELISA-based dot blot (**A** and **C**) and Western blot (**B**) at 24 (**A** and **B**) and 72 (**C**) hours post infection. Panel D shows concentration of aquaporin 5 (Western blot) and MMC6 antigen (ELISA-based dot blot) in lung tissue after 24 and 72 hours. Panel A: Control $n = 3$, D39 10^8 $n = 7$, D39 10^9 $n = 5$; Panel B: $n = 3$ for all groups; Panel C: Control $n = 9$, 24 hours D39 10^9 $n = 5$, D39 10^9 72 hours $n = 4$; Panel D: MMC6 antigen; Control $n = 4$, 24 hours D39 10^9 $n = 3$, 72 hours D39 10^9 $n = 4$; Aquaporin 5; $n = 3$ for all groups.

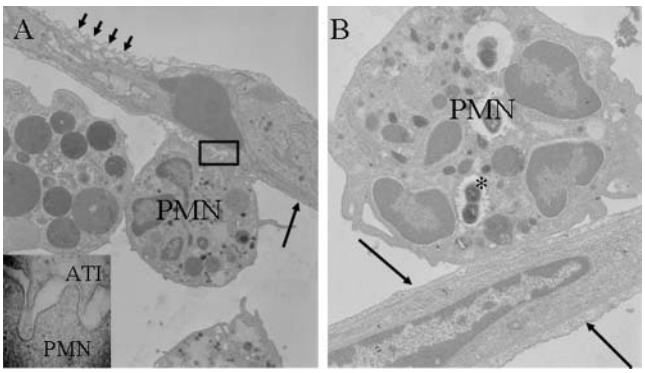

FIGURE 3 Ultrastructure of alveolar epithelial Type I cells 24 hours post infection with *S. pneumoniae* (D39). Lung samples selected from areas that were macroscopically inflamed at *post mortem*. Panels A and B show electron micrographs of airspaces containing polymorphonuclear cells (PMN). In some areas, alveolar Type I (ATI) cells appear oedematous and have ruffled apical plasma membranes (small arrows) while in other areas ATI plasma membranes appear normal (long arrows). ATI cell membrane also observed interdigitating with the plasma membrane of an adjacent neutrophil (insert magnified from box shown in A). Star (∗) bacteria present in PMN vacuole.

Electron microscopic assessment

Macroscopically inflamed regions were selected for examination by electron microscopy. In these regions, ATI cells were intact although the plasma membrane was ruffled in places (Figure 3).

Immunofluorescence assessment

To further confirm that the decrease in lung RTI_{40}/podoplanin concentration was not associated with ATI cell necrosis, in *S. pneumoniae*-infected lungs, ATI cells were imaged with anti-RTI_{40}/podoplanin antibody [22] and a new monoclonal antibody against ATI cells, MMC6 (unpublished data, McElroy and Dobbs, 1995). The MMC6 antibody is a different isotype to that of RTI_{40}/podoplanin and therefore can be used simultaneously with RTI_{40}/podoplanin under the same imaging conditions (Figures 4 and 5). Confocal images demonstrated that RTI_{40}/podoplanin expression (green) was decreased throughout the lung 24 hours post *S. pneumoniae* infection (i.e., in both macroscopically inflamed and non-inflamed regions). In addition, in places there was focal loss of RTI_{40}/podoplanin staining at both low (Figure 4) and high (Figure 5) power. In contrast, MMC6 staining (red) in *S. pneumoniae*-infected lungs was largely unchanged to that of control lungs (Figures 4 and 5). These images suggest that the decrease in lung RTI_{40}/podoplanin concentration was not associated with ATI cell necrosis.

Biochemical assessment of aquaporin 5 and MMC6 antigen

To determine whether other ATI cell-selective proteins were also down-regulated following instillation of *S. pneumoniae*, the concentration of aquaporin 5 and MMC6 antigen were determined in lung tissue using ELISA-based assays (Figure 2D). The concentration of both these proteins was decreased in lung homogenates by approximately 70% at 24 hours post infection (Figure 2D). By 72 hours post infection, the lung concentration of both proteins was increased compared with values at 24 hours post dose although still lower than control values. Western blot analysis confirmed the decrease of aquaporin 5 in lung tissue. The MMC6 monoclonal antibody does not recognize the denatured antigen and therefore was not identified by Western blot analysis. These data demonstrate that other ATI cell-specific proteins are decreased following *S. pneumoniae* infection.

ATII Cell Analysis

Electron microscopic and immunofluorescence assessment

Electron microscopy and confocal imaging (with type II AEC-selective antibodies) were also performed to determine the extent of ATII cell injury in response to *S. pneumoniae* infection (Figure 6). Electron microscopic analysis demonstrated sloughing of ATII cells into the airspaces (Figure 6B); this sloughing was not seen in control lungs (Figure 6A). Confocal imaging with antibodies against $RTII_{70}$ and MMC4 (identified as aminopeptidase N (APN); unpublished data, Franklin and McElroy, 2004), to identify the ATII cells phenotypically, also demonstrated shedding of ATII cells into the airspaces. Quantification of $RTII_{70}$/MMC4 positive cells (i.e., ATII cells) in the process of being sloughed from the alveolar wall and/or in the airspaces demonstrated a significant increase 24 hours post infection compared with controls. At 72 hours post infection, the number of ATII cells in the airspaces was still increased over control values but less than at 24 hours post infection.

Biochemical assessment

Further studies were performed to determine whether the extent of ATII cell injury could be determined biochemically (Supplementary Data 2 - available online). SP-D (expressed by both ATII and Clara cells) was increased in BALF and lung homogenates at 24 and 72 hours post infection. Lung concentration of pro-SP-C (which is specific for ATII cells) decreased with dose at 24 hours post infection.

Therefore, the combined morphologic and biochemical data suggest that *S. pneumoniae* causes some

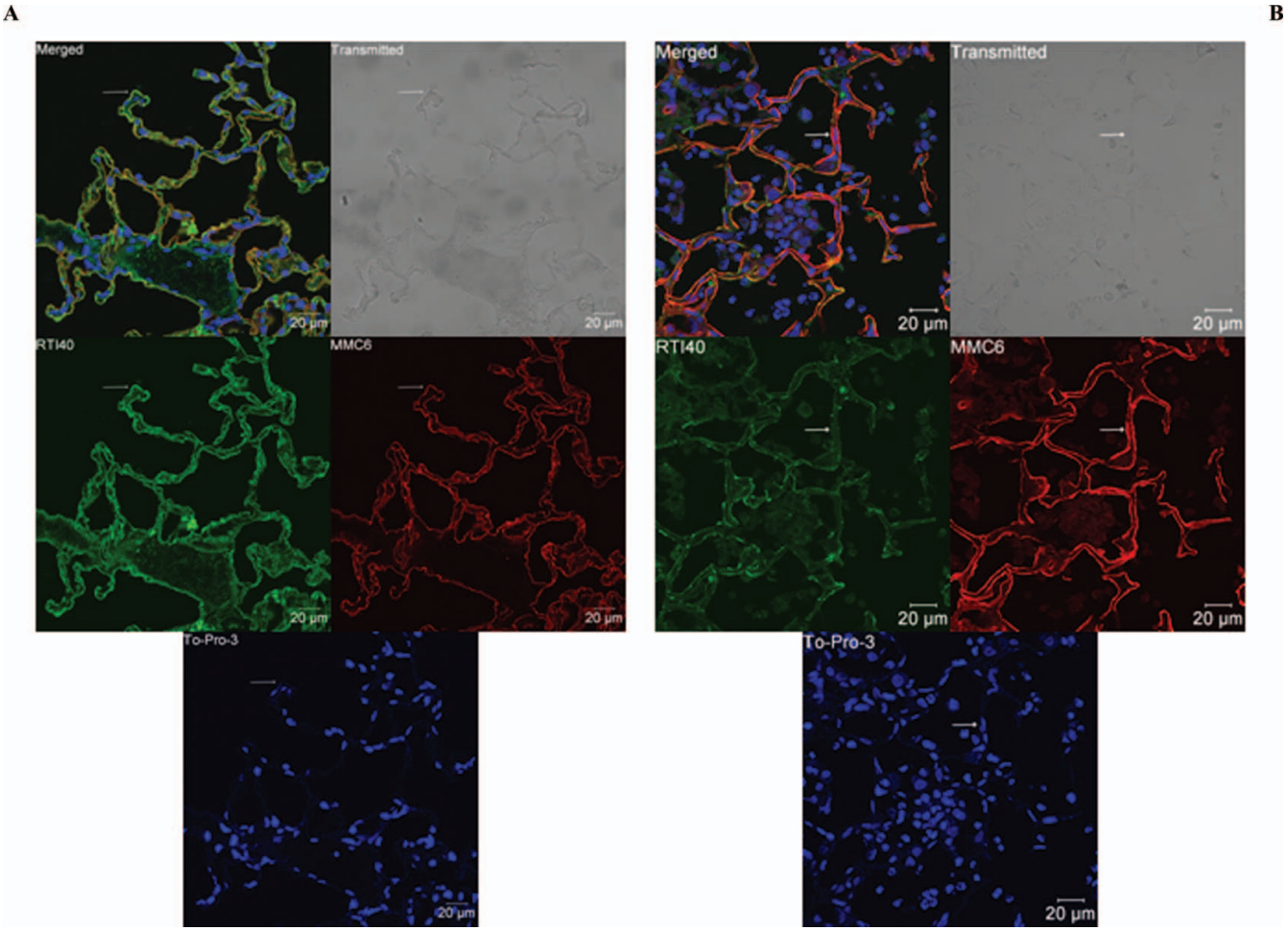

FIGURE 4 Confocal imaging to demonstrate decreased expression of RTI_{40} in alveolar epithelial cells 24 hours post *S. pneumoniae* infection; low power image. Control (**A**) and infected (**B**) lungs were stained with 2 antibodies specific for ATI cells; RTI_{40} (green) and MMC6 (red). Nuclear stain To-Pro-3 is shown in blue. Merged signal is yellow demonstrating a co-localized signal from RTI_{40} and MMC6. Increased numbers of nuclei in infected lungs are due to an increased number of inflammatory cells in the airspaces. Representative images from $n = 3$.

ATII cell injury but not sufficient to prevent the increase in BALF and lung SP-D levels.

DISCUSSION

We have investigated the host response to *S. pneumoniae*-induced pneumonia in terms of AEC injury and cell-selective protein expression, and have illustrated the following findings:

- Novel down-regulation and/or degradation of the ATI cell-selective protein RTI_{40}/podoplanin in *S. pneumoniae*, independent of ATI cell necrosis.
- Identification and quantification of cells within the alveolar airspace co-expressing ATII cell-selective markers $RTII_{70}$ and MMC4/APN, consistent with ATII cell sloughing in *S. pneumoniae*.
- Combined biochemical and morphologic data suggesting that distal instillation of *S. pneumoniae* leads to ATII cell injury but not ATI injury.

ATI Cell-specific/Selective Protein Expression

In various models of experimental acute lung injury, the amount of RTI_{40}/podoplanin recovered in BALF is associated with morphologic and functional damage to the alveolar epithelium [12–14]. However, here we demonstrate that both lung and BALF concentrations of RTI_{40}/podoplanin are dramatically down-regulated post *S. pneumoniae* infection. This reduction in RTI_{40}/podoplain was not associated with morphologic injury to ATI cells, as shown by ultrastructural examination of ATI cells and by confocal microscopy with an alternative apical surface ATI cell antigen (MMC6). In addition, confocal

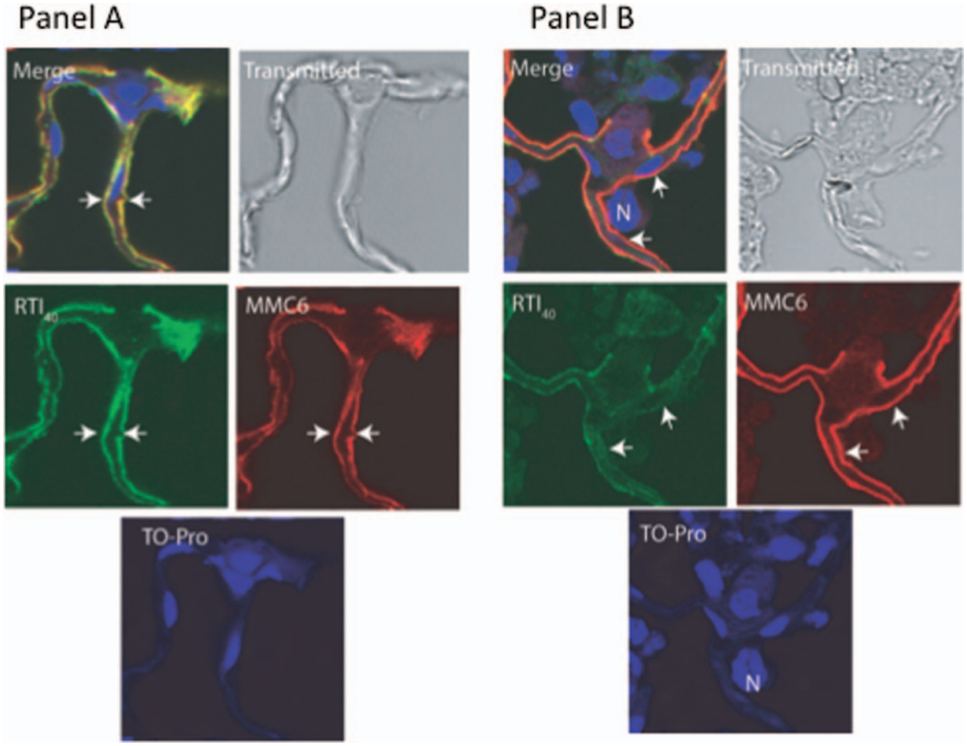

FIGURE 5 Confocal imaging to demonstrate decreased expression of RTI_{40} in alveolar epithelial cells 24 hours post *S. pneumoniae* infection; high power image. Control (**A**) and infected (**B**) lungs were stained with 2 antibodies specific for ATI cells: RTI_{40} (green) and MMC6 (red). Nuclear stain To-Pro-3 is shown in blue. Merged signal is yellow demonstrating a co-localized signal from RTI_{40} and MMC6. Arrows in (**B**) demonstrate focal loss of RTI_{40} on the surface of ATI cells. Increased numbers of nuclei in infected lungs are due to an increased number of inflammatory cells and ATII cells in the airspaces (N = neutrophil). Representative images from $n = 3$.

microscopy demonstrated that RTI_{40}/podoplanin was reduced throughout the lung even in areas that were not microscopically inflamed. Therefore, these data suggest that the phenotype of ATI cell can change in response to injury/inflammation and that this response may be a general "host" response since it was not restricted to inflamed regions.

To determine whether the reduction in lung RTI_{40}/podoplanin was a selective or general ATI cell response, we also measured the lung concentration of other ATI cell marker proteins (i.e., aquaporin 5 and MMC6). Our data demonstrate that the lung concentration of both these proteins was decreased at 24 hours post infection and the extent of this increase was similar to RTI_{40}/podoplanin. However, with regard to the expression of MMC6, decreased lung concentration was not associated with a reduced expression on the surface of ATI cells. These data suggest, at least for the MMC6 antigen, that decreased lung concentrations may be secondary to dilution with oedema fluid and not necessarily specific down-regulation on the surface of ATI cells as observed for RTI_{40}/podoplanin.

The observation that the concentration of RTI_{40}/podoplanin was decreased in both BALF and lung tissue, in the absence of widespread morphologic injury to ATI cells, was unexpected. Using a similar ELISA-based dot blot assay, Frank and colleagues demonstrated increased concentrations of RTI_{40}/podoplanin in BALF and plasma in an acid-induced lung injury model [23]. However, we did not detect RTI_{40}/podoplanin in plasma in this study (data not shown) suggesting the protein was not cleared into blood and consistent with the observation that ATI cells were largely morphologically intact (i.e., no ATI cell membranes shed). RTI_{40}/podoplanin immunoreactivity was reduced on the surface of ATI cells in an experimental model of lung fibrosis [24]. However, unlike the loss of immunoreactivity observed here, RTI_{40}/podoplanin concentrations were elevated in BALF in this model of lung fibrosis [24]. In human cell lines, RTI_{40}/podoplanin expression is regulated at both the transcriptional and translational level [25]. It is therefore possible that the reduced expression on ATI cells is specifically down-regulated in response to *S. pneumoniae* infection although

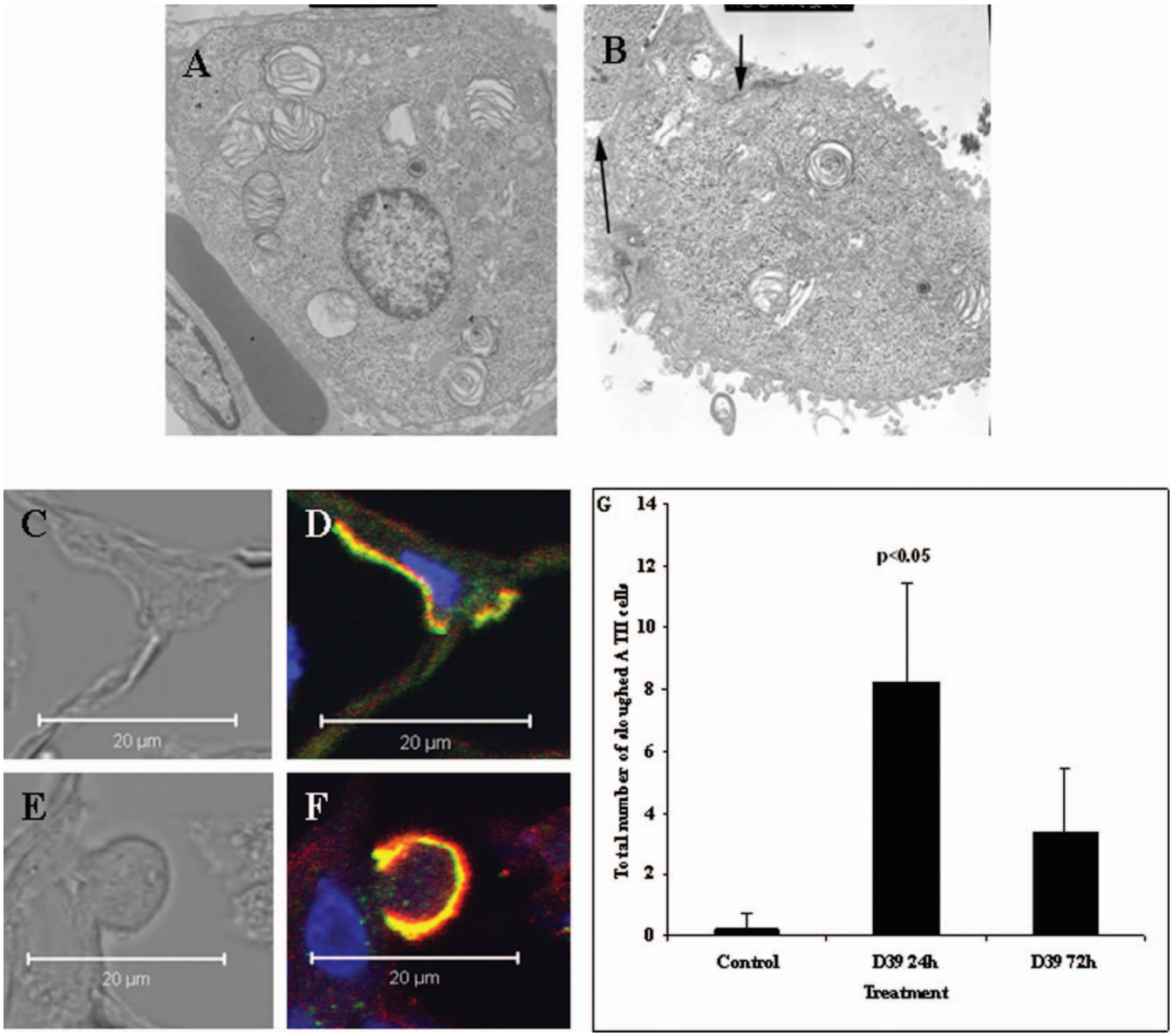

FIGURE 6. Identification and quantification of sloughing ATII cells in *S. pneumoniae* (10^9 CFU)-infected lung tissue. (**A** and **B**) Panels show EM of **A** normal (original magnification × 10,000) and **B** sloughing (original magnification × 8000) phenotypes of ATII cells 24 hours after infection with *S. pneumoniae*. Arrows in image **B** illustrate cell sloughing away from the basement membrane. (**C**–**F**) Panels show transmitted (**C** and **E**) and immunofluorescence (**D** and **F**) images of normal (**C** and **D**) and sloughing (**E** and **F**) phenotypes (original magnification × 40) of ATII cells 24 hours post infection with *S. pneumoniae*. Immunofluorescence (**D** and **F**) shows staining of ATII cells with $RTII_{70}$ (green), MMC4/APN (red), and nuclear stain To-Pro-3 (blue). (**G**) Panel shows quantification of sloughing cells after infection with *S. pneumoniae*. Images representative of $n = 3$.

we did not investigate the mechanism in this study.

The role of RTI_{40}/podoplanin is not known in adult lung but it is required for normal lung development since mice with RTI_{40}/podoplanin gene (TIα) deletion die after birth from respiratory distress [26]. However, the reduction in RTI_{40}/podoplanin observed here was not considered pathologically since the rats recovered after *S. pneumoniae* infection and the lungs returned to a normal morphology 3 weeks post dose. In cultured cells, overexpression of RTI_{40}/podoplanin promotes cell migration [27]. However, it is difficult to image how a reduction in the apical expression of RTI_{40}/podoplanin might reduce cell migration and how this might be non-pathological in an inflammatory environment. The C-type lectin receptor (CLEC-2) is an endogenous ligand for podoplanin and CLEC-2/podoplanin

interactions have been identified as important for platelet aggregation on the surface of tumor cells [28, 29]. Although the role of platelets in acute lung injury is not well understood [30], it is possible that reduced expression of RTI_{40}/podoplanin on the surface of ATI cells may prevent unnecessary thrombus formation (by limiting CLEC-2 platelet interactions). CLEC-2 is also expressed on neutrophils and activation of CLEC-2 is associated with neutrophil activation [15]. It is therefore also possible that RTI_{40}/podoplanin on ATI cells might function as an endogenous ligand for CLEC-2 on the surface of neutrophils; the reduction of RTI_{40}/podoplanin on ATI cells might serve to reduce the chances of neutrophil activation and thereby help limit the harmful effects of inflammation. Therefore, while the functional significance of reduced RTI_{40}/podoplanin expression on the surface of ATI cells following *S. pneumoniae* infection is unknown, our data suggest that this reduction is not pathological since the lungs return to normal morphology post infection.

ATII Cell Specific/Selective Protein Expression

As there are currently no specific biochemical methods to quantify ATII cell injury, we used a confocal assay to detect cells co-expressing $RTII_{70}$ and MMC4/APN. At 24 hours, a sub-population of ATII cells adopted a sloughing phenotype, in some cases detaching from the basement membrane completely. Although pneumococcal products have been shown to directly damage AECs in vitro [31, 32], this is the first time that specific damage to ATII cells (in the absence of ATI cell injury) has been identified in a clinically representative in vivo model. Here, we observed dissemination of *S. pneumoniae* into the blood of some, but not all infected animals (data not shown). It is possible that dissemination could have occurred across the alveolar membrane in association with sloughing ATII cells. However, although we detected ATII cell damage, the extent of damage was not sufficient to prevent normal lung repair since the lungs had a normal morphology 3 weeks post infection.

We also determined the concentration of lung pro-SP-C in BALF and lung homogenates. While we did not detect pro-SP-C in BALF, lung concentrations of pro-SP-C were decreased following *S. pneumoniae* infection. This decrease is consistent with our morphologic data demonstrating loss/damage of ATII cells. Here, we also demonstrate that SP-D is dramatically increased in lung and BALF following *S. pneumoniae*. This increase is consistent with the role SP-D is known to have in the host response against *S. pneumoniae* [33, 34]. It would therefore seem likely that the increase in lung SP-D found here is important for the clearance of *S. pneumoniae* from the lungs.

CONCLUSION

It is widely believed that ATI cells are more easily damaged than ATII cells following the inhalation of harmful substances and that damage to ATII cells is potentially more harmful because ATII cells are required for alveolar epithelial repair. However, here we demonstrate the contrary. Following *S. pneumoniae* infection, we found evidence of ATII cell injury but not ATI injury suggesting that *S. pneumoniae* and/or the host response to *S. pneumoniae* are important causes of ATII cell injury in this model. We also demonstrate the dramatic reduction of RTI_{40}/podoplanin on the surface of intact ATI cells and hypothesize that RTI_{40}/podoplain-CLEC-2 interactions (or lack of interaction) might be important for the regulation of platelet aggregation and/or neutrophil activation in experimental acute lung injury.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary Material Available Online:

Supplementary Figure 1- Histology
Supplementary Figure 2- Type II AEC/Clara cell-specific markers in D39 S. pneumoniae-induced pneumonia